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Bovine CD49 positive-cell subpopulation remarkably increases in mammary epithelial cells that retain a stem-like phenotype

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ABSTRACT

We previously proved that adult stem cells reside in the bovine mammary gland and possess an intrinsic potential to generate a functional mammary outgrowth. The aim of this study was to investigate on the immunophenotyping features retained by mammary stem-like cells detected in long term culture. Flow cytometry analysis showed different subpopulations of mammary epithelial cells emerging according to the timing of cell culture. CD49f^{+}-cells significantly increased during the culture ($p < 0.01$) and a similar trend was observed, even if less regular, for CD29^{+} and ALDH1 positive cell populations. No difference during the culture was observed for CD24 positive cells but after 35 days of culture a subset of cells, CD49f positive, still retained regenerative capabilities in in vivo xenotransplants. These cells were able to form organized pseudo-alveoli when transplanted in immunodeficient mice. These results prove the presence of a multipotent cell subpopulation that retain a strong epithelial induction, confirmed in in vivo xenotransplants with a presumable in vitro expansion of the primitive population of adult mammary stem cells.

1. Introduction

Adult stem cells are presumed to exist during the entire life cycle of mammary glands, where they are required to expand the cell populations during pregnancy and sustain cell turnover to replace senescent cells. Stem cells are generally defined as cells displaying a self-renewal capacity either with or without differentiation, depending on the type of division. The symmetric division of stem cells produces two identical stem cells, resulting in the expansion of the stem cell population, whereas an asymmetric division will result in a new stem cell and a progenitor cell of a more committed lineage. These adult stem cells are generally considered long-lived, mostly quiescent, slow cycling cells that generate new stem cells, hereby maintaining the stem cell pool (Borena et al., 2013).

In order to study the functional properties of stem cells, it is necessary to identify and prospectively purify them, a task that has proven technically difficult because of the low numbers of stem cells in the tissue of origin, and the lack of universal morphologic traits for stem cells (Blau et al., 2001). Most stem-cell enrichment protocols rely on immunosorting and use sets of antibodies against cell-surface proteins. Current methods for detecting bovine mammary progenitors require the preparation of viable single-cell suspensions and their assessment in suitable in vitro or in vivo assays to detect the growth and differentiation properties of the input cells at a clonal density (Stingl et al., 2006). However, the efforts to purify adult stem cells from the bovine mammary gland have been hindered by the lack of cell-surface markers specific for undifferentiated or differentiated mammary cells (Martignani et al., 2009) even if suitable in vitro and in vivo assays for testing stem cell properties have been proposed by our laboratory (Martignani et al., 2010).

In rodents and human, primary cultures of mammary epithelial cells underwent a limited replication and rapidly differentiated in a process regulated by hormonal factors, extracellular matrix, and cell–cell interactions (Muschler et al., 1999; Reynolds and Weiss, 1996; Romanov et al., 2001; Simian et al., 2001). We have recently demonstrated that it is possible to maintain bovine epithelial cells with stem-like properties in long-term culture under appropriate culture conditions that maintain a multipotent cell subpopulation with an intrinsic regenerative potential (Cravero et al., 2014), but no further data are available to understand the mechanism involved in the regulation of the bovine mammary stem niche if those are the remaining part of stem cells already present in the culture since the beginning of the culture or if it is possible to renew the pool during the culture time. This fact is of interest because it may prove the expansion and enrichment of a stem cell subpopulation in vitro. In humans, it has previously demonstrated that non-adherent mammospheres are enriched in cells with functional characteristics of stem/progenitor cells that may be a feasible method to isolate and characterize mammary stem cells (Tosoni et al., 2012), however the loss of the paracrine context with other cell types does not explain the causes which allow the presence and maintenance of the mammary stem cell niche (Dontu et al., 2003).

The aim of this work was to analyze the surface antigen expression and functional features of cell populations in a long-term mammary

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cell culture that maintains the regenerative properties. The ability to sustain in long-term culture a stem cell niche should give interesting perspectives for the study of their proliferation and differentiation and a new tool for their manipulation.

2. Materials and methods

2.1. Bovine mammary tissue

The bovine mammary tissue was collected from slaughterhouse from 5 to six years old cows. Sample collection was performed with the authorization and under the supervision of representatives of the Veterinary Services of the Italian National Health Service branch of the Ministry of Health as previously described (Martignani et al., 2009). Briefly, the tissue was minced, transferred to a 125 ml tube containing 20 ml of a 1:1 v/v mixture of Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 Ham (DMEM/F12) supplemented with 2% w/v bovine serum albumin, 300 U/ml collagenase, 100 U/ml hyaluronidase, 100 U/ml penicillin, 100 mg/ml streptomycin (all from Sigma Aldrich, St. Louis, MO, USA). After 18–20 h of incubation, a fraction enriched in epithelial cell aggregates (organoids) was next obtained by centrifugation and stored at −80 °C until further processed. To prepare single cell suspensions, organoids were thawed and incubated with a 0.5 mg/ml trypsin solution supplemented with 0.2 mg/ml EDTA and subsequent washing in Hank's balanced salt solution (HBSS, STEMCELL Technologies) supplemented with 2% FBS. Cells were then treated with 5 mg/ml dispase and 100 mg/ml DNAseI (Sigma Aldrich) and passed through a 40 μm cell strainer (BD Biosciences, San Jose, CA, USA) to remove remaining cell aggregates.

2.2. Cell culture

60 mm tissue culture dishes were coated with collagen by incubation for 1 h at 37 °C with a solution of rat tail type 1 collagen (80 μl of rat tail type I collagen 1.5 mg/ml diluted in 50 ml of PBS). Dissociated cells were cultured in SF7 Medium (0.1% BSA, 10 ng/ml EGF, 10 ng/ml Cholera Toxin, 1 μg/ml Insulin, 0.5 μg/ml Hydrocortisone, DMEM/F12 v/v). Cells were studied from P0 (from dissociation to the first passage) to P5 splitting them every 7 days. For immunostaining, 5×10^4 cells were plated in each well of a collagen coated 96-wells plate and fixed at passage 0, 1, 3 and 5 (see below the used fixative).

2.3. Colony-forming cell (CFC) assay

Single cell suspensions of bovine epithelial cells were added in a number of 500 cells/plate along with 2×10^5 NIH 3T3 mouse fibroblasts previously treated with 10 μg/ml mitomycin C (Sigma-Aldrich) for 2 h. Cells were cultured in human EpiCult B medium (StemCell Technologies) supplemented with 5% FBS, 10^{-6} M hydrocortisone (Sigma-Aldrich), 100 U/ml penicillin and 100 mg/ml streptomycin. The dishes were then incubated at 37 °C with 5% CO_2 for 24 h. The medium was then replaced omitting FBS. Cells were incubated for further 6–9 days and then the cultures were fixed with acetone/methanol (1:1 v/v, Fluka) and either stained with a crystal violet solution (50 mg crystal violet in a 20% methanol solution, Sigma-Aldrich) or immunostained with antibodies to human cytokeratin 14 (CK14), cytokeratin 18 (CK18=), and p63, after validation that all of these cross-reacted with bovine antigens. Colonies containing more than 50 cells (after 7 days of culture) or than 100 cells (after 10 days of culture) were then counted and progenitor frequencies were expressed as the total number of colonies obtained per 100 cells.

2.4. Immunostaining

Selected culture dishes were processed for immunostaining as described in (Martignani et al., 2010). The medium was removed from the dishes, and the cells were fixed with a 1:1 v/v mixture of acetone and methanol for 1 min. Cells were then washed and blocked with Tris–HCl buffered saline (0.1 M Tris HCl, 0.14 M NaCl, pH 7.6) supplemented with 10% goat serum (all reagents from Sigma-Aldrich Corp). Dishes were then incubated with primary antibodies for 1 h at room temperature followed by another hour with secondary fluorochrome-conjugated antibodies. The nuclei were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) at a concentration of 0.5 μg/ml. The primary antibodies used were an anti-human cytokeratin 14 (CK14, 1:500 dilution, polyclonal AF-64, Covance, Princeton, NJ, USA) and an anti-bovine CK18 (1:200 dilution, clone KS-B17.2, Sigma-Aldrich), p63 (1:200 dilution, clone 4A4, Thermo Fisher Scientific, Fremont, CA, USA), milk proteins (1:500 dilution, polyclonal Nordic Immunology, Tilburg, Netherlands) EpCAM (1:100 dilution, clone E144, AbCAM, Cambridge, UK). The primary antibodies used were: anti-human cytokeratin 14 (CK14, 1:500 dilution, polyclonal AF-64, Covance, Princeton, NJ, USA); anti-bovine CK18 (1:200 dilution, clone KS-B17.2, Sigma-Aldrich); p63 (1:200 dilution, clone 4A4, Thermo Scientific, Fremont, CA, USA); milk proteins (1:500 dilution, polyclonal Nordic Immunology, Tilburg, Netherlands); EpCAM (1:100 dilution, clone E144, AbCAM, Cambridge, UK). Secondary antibodies used were AlexaFluor® 488-labeled goat anti-rabbit IgG and AlexaFluor® 594-labeled goat anti-mouse IgG (both from Life Technologies, Carlsbad, CA, USA). Negatively stained controls were performed for each antigen by replacing the primary antibody with a suitable isotype (normal mouse IgG or normal rabbit IgG from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at the same concentration.

2.5. Flow cytometry

After picking the colonies from the CFC assays, cells were digested with warm (37 °C) trypsin (Sigma-Aldrich Corp) for 2 min while pipetting. Trypsin was subsequently neutralized with cold HBSS supplemented with 2% FBS. The resulting single cell suspension was then stained for flow cytometry. Staining for aldehyde dehydrogenase I (ALDH1) was done with ALDEFLUOR™ kit (STEMCELL Technologies) as per manufacturer's instruction. Cells were incubated for 30 min at 37 °C with the ALDEFLUOR substrate and then stained with an R-PE conjugated anti-human CD49f (1: 25 dilution in 50 μl volume, clone GoH3, Santa Cruz Biotechnology Inc., Dallas, TX, USA), FITC conjugated anti-human CD24-FITC or FITC conjugated antihuman CD29-(ImmunoTools GmbH, Germany). DAPI was then added at a concentration of 0.25 μg/ml in order to discriminate live from dead cells. Cells were then run on an Attune® Cytometer (Life Technologies) equipped with a 405 nm and a 488 nm laser. CD49f + cells were isolated using a FACSVantage SE (Beckton Dickinson).

2.6. Xenotransplants

Ten female NOD/SCID mice were bred and housed at the animal facility of the Department of Veterinary Science of the University of Turin according to the procedures and guidelines approved by the Italian Ministry of Health (n. 72 del 27/04/2011). Animal work described in this study has been reviewed and approved by the Italian Ministry of Health. Mice were used at 5 to 10 weeks of age as equivalent recipients for the transplants described (Prpar et al., 2012). Concentrated rat tail collagen was prepared as previously described (Richards et al., 1983). Collagen gels were prepared as previously described (Martignani et al., 2009). Each gel contained 1.6×10^5 10T1/2 fibroblasts previously treated with 2 μg/ml mitomycin C and 5×10^4 bovine primary mammary cells. At first a 2 cm anterior-to-posterior cut was made through the skin along a median line followed by a smaller incision of approximately 4–5 mm in the abdominal wall directly above the kidney position. The collagen gels were inserted under the kidney capsule using fire polished glass Pasteur pipettes. The abdominal wall was then sutured and the procedure was repeated on the controlateral kidney. A slow-release